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Original article

A Validated HPLC Method for Quantitation of Rosmarinic Acid in a Polyherbal Syrup

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Abstract

Background and objectives: "Monzej-e Soda" is a poly herbal decoction which is used in Iranian traditional medicine for many diseases especially the ones with similar signs to depression. In this investigation, an HPLC method for quantitation of rosmarinic acid as a marker of the "Monzej-e Soda" syrup has been developed and validated. **Methods:** The syrup was prepared by decocting a mixture containing *Lavandula angustifolia*, *Melissa officinalis*, *Echium amoenum*, *Cordia myxa*, *Glycyrrhiza glabra*, *Ziziphus jujuba*, *Foeniculum vulgare*, *Fumaria parviflora*, *Adiantum capillus-veneris* and *Alhagi* spp. Manna. Then, an HPLC technique was designed for analysis of rosmarinic acid in the syrup using C_{18} column (4.6 × 250 mm, 5 µm); H_3PO_4 0.085%: acetonitrile as the mobile phase in gradient mode. The flow rate, column temperature, wave length, injection volume and run time were 1 mL/min, 40 °C, 330 nm, 20 µL and 34 min, respectively. The method was validated for selectivity, linearity, precision, accuracy, LOD and LOQ. **Results:** By using the proposed method, rosmarinic acid was completely separated from the other components in the syrup chromatogram with acceptable peak shape. The concentration of rosmarinic acid in the syrup was found 47.5 mg/100 mL. The HPLC method was valid according to selectivity, linearity (72-110 µg/mL, r^2: 0.9995), intra-day and inter-day precisions (RSD%<2), accuracy (103.38-106.47%), LOD 1.6 µg/mL and LOQ 4.9 µg/mL. **Conclusion:** This method is a simple and suitable one for quantitation of rosmarinic acid in "Monzej-e Soda" syrup and could be used for quality control of the syrup.

Keywords: Herbal; HPLC; Iranian traditional medicine; Monzej-e Soda; Rosmarinic acid; Validation

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Introduction

The role of medicinal plants in the treatment of various mental disorders has recently become widespread [1,2]. Plants may provide alternatives to synthetic antidepressants with more safety and efficacy [3]. Herbal remedies can be used alone or in combination with other herbs. Despite chemical drugs, medicinal herbs and their preparations show synergistic effects

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due to their multi-components and they could be a good alternative to producing new drugs [4,5]. Iranian traditional medicine (ITM) or Persian medicine has been used for prevention and treatment of various diseases for thousands of years. It uses various polyherbal combinations for diverse diseases. One of the herbal mixtures in ITM is "Monzej-e soda" which is composed of ten herbal components including *Lavandula angustifolia* Mill., *Foeniculum vulgare* Mill., *Ziziphus jujuba* Lam., *Alhagi* spp. Fisch. manna, *Echium amoenum* L., *Cordia myxa* L., *Glycyrrhiza glabra* L., *Melissa officinalis* L., *Fumaria parviflora* Lam. and *Adiantum capillus-veneris* L. that is used for treatment of a wide range of mood disorders with similar signs to depression [6]. This mixture should be decocted and used for at least two weeks. Herbal combinations which are used in traditional systems as decoction can be converted to syrup which has better acceptance. On the other hand, according to the World Health Organization (WHO) principles, the active herbal ingredients must be determined using standard techniques before performing clinical trials. Because natural products usually have various complex compounds and their quality control is difficult, modern quality control methods are needed to overcome this problem [7]. In a complex herbal mixture, in addition to common tests, a marker is usually selected for quality control of the product. In "Monzej-e soda" mixture, most of the herbal ingredients contained phenolic compounds and rosmarinic acid is the one of the phenolics (figure 1) which is dominant in some of the herbals of the mixture including *Lavandula angustifolia* [8-10], *Foeniculum vulgare* [11-13], *Melissa officinalis* [10, 14-17], *Echium amoenum* [18,19] and *Adiantum capillus-veneris* [20]. Rosmarinic acid is common in many species of herbs, particularly in the families of Boraginaceae and Lamiaceae [21] and has been revealed to have various effects such as antioxidant, anti-inflammatory, antimicrobial and antiviral properties [22, 23]. It has demonstrated antidepressant effect in the forced swimming test in mice [24-26] and displayed anti-allergenic, anti-thrombotic and anti-carcinogenic effects as well [25]. Regarding to the biological effect of rosmarinic acid, it could be considered as a suitable marker for quality control of the "Monzej-e soda" mixture. Moreover, introducing a validated method for determination of rosmarinic acid in the product is required. Different techniques are used for determination of herbal compounds in mixtures or products. High performance liquid chromatography (HPLC) is a popular analytical method for quantitation of herbal medicine ingredients due to high detection sensitivity, separation efficiency and selectivity [27]. Therefore, it is a suitable method for quantitation of rosmarinic acid in "Monzej-e soda" syrup. In the present investigation, an HPLC method for quantitative determination of rosmarinic acid in "Minzej-e Soda" syrup as a polyherbal product was developed and validated according to selectivity, linearity, precision, accuracy, LOD and LOQ.

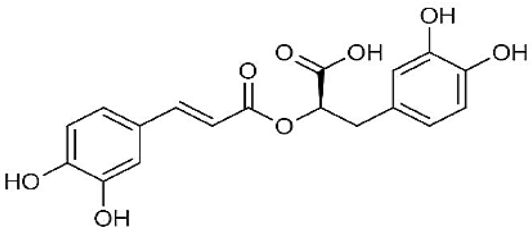

Figure 1. Chemical structure of rosmarinic acid

Materials and Methods

Ethical considerations

The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran with the code No. IR.SBMU.RETECH.REC.1396.532.

Chemicals

Rosmarinic acid standard material, potassium sorbate (Sigma-Aldrich, USA), glycerin (Merck, Germany) and HPLC solvents (Dukson, South Korea) were applied in the study. Solvents used were of analytical grade.

Instrumentation

The analytical HPLC experiment was performed using an Agilent Technologies equipped with a vacuum degasser, auto-sampler and a UV detector. The column used was an ACE, C_{18} HL (4.6 × 250 mm, 5 µm). The mobile phase was H_3PO_4 (0.085%): acetonitrile as gradient mode (table 1) for 34 min. The flow rate was 1 mL/min. Volume of Injection for all samples and standard solutions was 20 µL. λ_{max} was 330 nm and the column temperature was kept at 40° C.

Table 1. Gradient program of the mobile phase

Time (min)	H_3PO_4 (0.085%)	Acetonitrile
0	84	16
10	84	16
25	78	22
26	10	90
29	10	90
30	84	16
34	84	16

Plant material

Plants required for preparation of the syrup were provided from an herbal market in Tehran in April 2016. The samples were identified by the botanists at the Herbarium of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. Herbal Market Samples (No. 465-474 HMS for *Lavandula angustifolia* Mill. aerial parts, *Foeniculum vulgare* Mill. fruits, *Ziziphus jujuba* Lam. fruits, *Alhagi* spp. Fisch. manna, *Echium amoenum* L. petals, *Cordia myxa* L. fruits, *Glycyrrhiza glabra* L. rhizomes, *Melissa officinalis* L. aerial parts, *Fumaria parviflora* Lam. and *Adiantum capillus-veneris* L. whole plant, respectively) were deposited at the Herbarium of TMRC for future references.

Formulation of the syrup

According to the ITM reference [6], *Lavandula angustifolia*, *Melissa officinalis*, *Echium amoenum*, *Fumaria parviflora*, *Foeniculum vulgare* and *Adiantum capillus-veneris* (1.9 g), *Cordia myxa* (3.6 g), *Glycyrrhiza glabra* (2.8 g) and *Ziziphus jujuba* (0.6 g) were powdered coarsely and extracted by using decoction method with distilled water for 30 min (plant: water 1:15 w/v). The mixture was filtered and concentrated. Then, the powdered *Alhagi* manna (3.6 g) was added to the mixture, shaked and filtered. Glycerin (16 mL) and potassium sorbate (0.2 g) were added to the mixture and the volume was adjusted to 100 ml with distilled water.

Determination of rosmarinic acid in the syrup

Sample preparation

Two mL of syrup was diluted to 10 mL with Ethanol: H_2O (3:2) in a volumetric flask.

Standard preparation

Stock solution of rosmarinic acid (0.110 mg/mL) was prepared in ethanol: H_2O (3:2). Different concentrations (0.072–0.110 mg/mL) were provided from the stock solution and used for plotting the calibration curve of rosmarinic acid.

Procedure

Sample and standard solutions were injected to HPLC system triplicate and rosmarinic acid content of the syrup was computed using area under the curve of standard and samples peaks of rosmarinic acid in the chromatograms.

Validation of the HPLC method

The HPLC method for quantitation of rosmarinic acid was validated through its selectivity, linearity, precision, accuracy/recovery, limit of detection (LOD) and limit of quantitation (LOQ).

Selectivity

Selectivity should be evaluated to differentiate and quantify the analyte in the presence of other components of the sample [28]. The chromatogram of the sample solution was analyzed and the peak of rosmarinic acid was assessed for peak shape and resolution from the nearest eluting peak. Moreover, placebo (a syrup containing glycerin and potassium sorbate) and diluent were injected to the HPLC and the chromatograms were compared to sample chromatogram to confirm the absence of any peak in the retention time of rosmarinic acid.

Linearity

Linearity is the potency of the technique to elicit test results that are directly proportional to the concentration of analytic within a certain range [29]. Linearity was considered via the relationship between the rosmarinic acid concentration and the response obtained from the UV-HPLC detector. Linearity was acquired by calibration curve from the solutions of rosmarinic acid (0.072–0.110 mg/mL) at four points with triplicate analysis to recognize the extent of the total variability of the response that could be explained by the linear regression model.

LOD and LOQ

LOD is described as the lowest analyte concentration in a sample that can be detected, not quantified. LOQ is explained as the lowest analyte concentration in a sample that can be quantified with acceptable precision. LOD and LOQ of the method were measured using $3.3\sigma/s$ and $10\sigma/s$ phrases, respectively, where σ is the standard deviation of the intercept and s is the calibration curve slope [30].

Precision

Consistent response of a measurement under stable conditions is defined as precision. Precision is variability measurement of the results, commonly explained by RSD% for number of samples which are statistically significant [31]. In the present study, three samples of the herbal syrup in three levels (80, 100, 120%) were analyzed for the intra-day and inter-day precisions. Each sample was injected to HPLC in triplicate on the same day and three consecutive days and RSD% was measured.

Accuracy

The similarity between experimental values and the real ones is known as accuracy (recovery). This parameter ensured that no missing or absorbance has occurred during the process. In the present investigation, recovery was calculated in 120%, 135% and 150% levels in the herbal syrup. Each sample was injected into HPLC three times.

Results and Discussion

The results obtained from method validation for rosmarinic acid quantitation showed that the suggested method was reliable. The high correlation coefficient value (r^2: 0.9995) demonstrated excellent linearity between areas of curves (AUC) and rosmarinic acid concentrations. The method was found linear at 72-110 µg/mL with equation y = 55.979.5x-208.9. LOD and LOQ (1.6 and 4.9 µg/mL) demonstrated high sensitivity of the method. Rosmarinic acid peak in sample was confirmed by comparison between retention times of standard solution and syrup peaks (figures 2). The concentration of rosmarinic acid in syrup was found 47.5 mg/100 mL. The peak starting and ending positions at the specific retention time demonstrated peak purity of rosmarinic acid. Peaks starting and ending positions also showed no interference of other components at rosmarinic acid peak and the obtained peak was completely separated from other components (figure 2). Results of precision and accuracy have been demonstrated in tables 2 and 3.

In assay of rosmarinic acid in the syrup, after comparison between different C_{18} columns with different lengths and particle sizes such as (4.6×100, 3.5µm); (4.6×150, 5µm) and (4.6×250, 5µm), maximum separation efficiency was achieved with the ACE, HL, C_{18} column, (4.6×250 mm, 5µm).

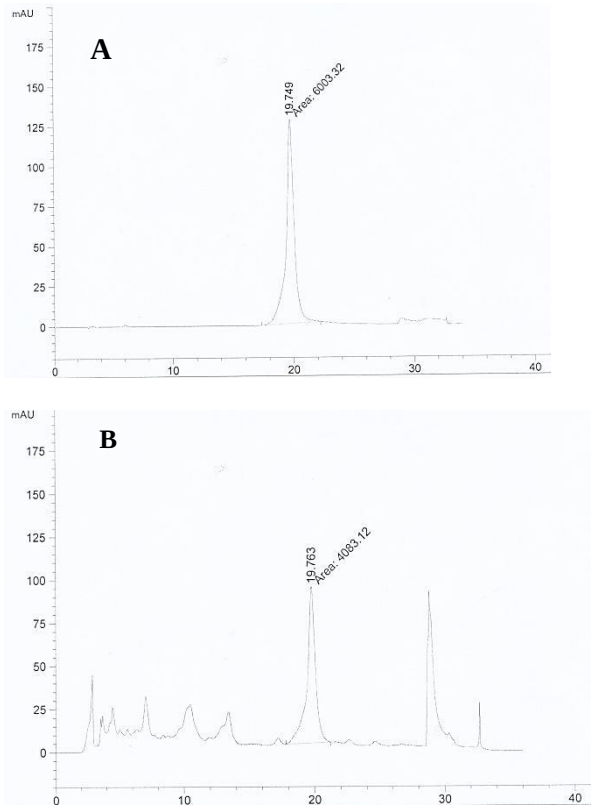

Figure 2. HPLC choromatogram of A) rosmarinic acid; B) polyherbal syrup

Table 2. Precision study of rosmarinic acid quantitation in "Monzej-e Soda" syrup

Days	Level	Rosmarinic acid content (mg/100mL)	Mean±SD (RSD%)	
			Intra-day	Inter-day
1	80%	37.10	37.55±0.69 (1.83)	80%: 37.81±0.25 (0.67) 100%: 47.55±0.70 (1.41) 120%: 56.61±0.94 (1.67)
		37.20		
		38.34		
	100%	47.46	48.48±0.94 (1.94)	
		48.65		
		49.32		
	120%	55.98	56.51±0.91 (1.60)	
		56.00		
		57.56		
2	80%	38.12	37.83±0.70 (1.84)	
		38.34		
		37.04		
	100%	46.29	47.02±0.68 (1.44)	
		47.63		
		47.14		
	120%	56.63	55.72±0.80 (1.43)	
		55.40		
		55.14		
3	80%	37.33	38.05±0.69 (1.81)	
		38.70		
		38.14		
	100%	47.06	47.15±0.69 (1.46)	
		46.52		
		47.88		
	120%	57.10	57.60±0.44 (0.76)	
		57.90		
		57.81		

Table 3. Recovery study of rosmarinic acid quantitation in "Monzej-e Soda" syrup

RA added	Found (mg/mL)	Recovery (%)	Mean±SD
0%	47.06	-	-
	46.52	-	
	47.88	-	
20%	59.39	104.96	106.47±1.42
	60.98	107.78	
	60.35	106.66	
35%	68.03	106.89	105.73±1.00
	66.93	105.15	
	66.93	105.15	
50%	72.94	103.13	103.38±0.72
	72.73	102.83	
	73.69	104.19	

The mobile phase consisting H_3PO_4 (0.085%): acetonitrile in gradient mode was chosen as the best one. Flow rates were set in 0.5, 1 and 1.5 mL/min. A flow rate of 1 mL/min reached an optimum signal/noise ratio with a suitable retention time (19.7 min) for rosmarinic acid. The results of rosmarinic acid determination method validation according to linearity, selectivity, recovery, and precision indicated that the suggested technique was appropriate for the quantitation of rosmarinic acid in the syrup.

In similar investigation on rosmarinic acid in a polyherbal decoction with some similarity with our syrup, a C_{18} column was used by gradient mode using 0.3% aqueous formic acid and 0.3% methanol formic acid. Flow and λ_{max} were 1.0 mL/min and 290 nm, respectively. This method was approved according to standard parameters and it was valid to determine the levels of rosmarinic acid and five other active components in examined product [32]. In another research, an HPTLC method for a polyherbal methanol extract was used. The components were separated on thin layer plate of silica gel as stationary phase, ethyl acetate:formic acid:acetic acid;water 15:1:1:1.5 as the mobile phase and trichloroethanol as the color reagent at 365 nm [33]. In another study rosmarinic acid contents of 29 species of Labiatae were determined by using HPLC method by using an ACE, C_{18}, (250 × 4.6 mm, 5µm) column. Mobile phase was 0.085% *O*-phosphoric acid in water, methanol and 2-propanol in gradient mode at 1.0 mL/min and 330 nm [34]. Also, a HPLC/DAD/ESI-MSn technique was developed for quantitation of the active compounds containing rosmarinic acid in GXN injection. The chromatography was carried out using an C_{18} column with a flow rate of 1.0 mL/min. Separation was achieved with a gradient program of methanol in water:formic acid (100:0.8) in three wavelengths 254, 280 and 320 nm [35]. Li et al. used a RP-HPLC method for the simultaneous separation and determination of 9 active components in ShuangDan (SD) oral liquid, a traditional Chinese medicine. Chromatography was done on a C_{18} column with gradient elution by methanol and 3% glacial acetic acid aqueous solution at a flow rate of 1.0 mL/ min. The method was enabled to analysis of 9 active components simultaneously [36]. Comparing to the above mentioned techniques, the current technique is a more simple method for rosmarinic acid quantitation with simple sample preparation and mobile phase and could be used for quality control of the syrup.

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Author contributions

Seyed Alireza Mortazavi, Homa Hajimehdipoor and Masoumeh Sabetkasaei supervised the study. Rasool Choopani and Shirin Fahimi were involved in traditional part. Sara Zakerin and Fatemeh Tavakolifar performed experimental section. All authors approved the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

ITM: Iranian traditional medicine; HPLC: high performance liquid chromatography; WHO: World Health Organization; LOD; limit of detection; LOQ: limit of quantitation; AUC: area under the curve.